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The impact of extracellular vesicles on parasite-host cell interactions: Searching for  
biomarkers and new anti-parasite strategies.

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27 ABSTRACT

28

29 Extracellular Vesicles (EVs) are released by a wide number of cells including blood cells,  
30 immune system cells, tumor cells, and adult and embryonic stem cells. EVs are an  
31 heterogeneous group of vesicles (~30-1000 nm) known by several different names  
32 including: microparticles, microvesicles, ectosomes, shedding vesicles or exosomes.  
33 The various roles of EVs during parasite-host cell interaction have been described  
34 recently in several diseases, bringing to the fore a novel concept in cell communication  
35 between parasites and host cells. The physiological release of EVs represents a normal  
36 state of the cell raising a metabolic equilibrium between catabolic and anabolic  
37 processes. Moreover, when the cells are submitted to stress with different inducers or  
38 in pathological situations (malignances, chronic diseases, infectious diseases.), they  
39 respond with an intense and dynamic release of EVs. The EVs released from stimulated  
40 cells versus those that are released constitutively may themselves differ, both physically  
41 and in their cargo. EVs contain protein, lipids, nucleic acids and biomolecules that can  
42 alter cell phenotypes or modulate neighboring cells. In this review, we have summarized  
43 ~~most of the~~ findings involving EVs in certain specific protozoan diseases. We have  
44 commented on strategies to study the communicative roles of EVs during parasite host  
45 cell interaction and hypothesized on the use of EVs for diagnostic, preventative and  
46 therapeutic purposes in infectious diseases. This kind of communication could modulate  
47 the innate immune system and reformulate concepts in parasitism. Moreover, the  
48 information provided within EVs could provide alternatives in translational medicine.

49

50

## 51 KEY FINDINGS

52 Extracellular vesicles (exosomes and microvesicles) have different biogenesis  
53 and can be released constitutively or under stimulation during host pathogen  
54 interactions.

55 Extracellular vesicles contain protein, lipids, nucleic acids and biomolecules  
56 that participate in cellular communication with other cells.

57 Extracellular vesicles have a potential diagnostic value.  
58

## 59 INTRODUCTION

60 Neglected diseases, including leishmaniasis and Chagas disease, cause many  
61 thousands of deaths per annum, and are prevalent in several regions of the world, but  
62 mainly in underdeveloped regions, associated with poverty, in Asia, Africa and the  
63 Americas (Hotez, 2017). The World Health Organization (WHO) have implemented  
64 various strategies to combat these diseases, including treatments, surveillance,  
65 improvement of housing, and vector control. These have reduced significantly the  
66 prevalence allowing prediction and may result in the elimination of some diseases, such  
67 as human African trypanosomiasis, in the next years (WHO, 2016).

68 Chemotherapy against protozoan parasites is a field that needs to improve and different  
69 current challenges are being considered. Several drugs have been used for decades,  
70 but they have limited efficiency, because of the development of drug resistance, toxicity  
71 issues for patients, and because their administration needs supervision thus incurring  
72 high costs for the health system (Klokousas et al, 2003; Hart et al, 2017; Menegon et al,  
73 2016). Hopefully, new antiprotozoan drugs will be made available in the next few years  
74 to follow the success of Artemisinin for malaria.

75 Efforts have been made in the post-genomic era to elucidate metabolic pathways in  
76 parasites with the aim of discovering specific targets that may be important for putative

77 chemotherapies and also to improve current drugs used as treatments (Lechartier et al,  
78 2014; reviewed by Weigelt, 2010).

79 The challenge for improving therapies is a better understanding of host-parasite  
80 interactions with an improved view on parasite adaptation and how the pathogen is able  
81 to manipulate its host environment. In recent decades, the research in this area has  
82 largely focused on the biomolecules secreted by parasites, many of which down-  
83 modulate host immune responses (reviewed in Evans-Osses et al, 2015). Despite the  
84 progress in this field and the description of secreted products in different parasitic  
85 infections (Kaur et al, 2001; Coakley et al, 2017; Grébaut et al, 2009), little is known  
86 about the mechanism(s) that regulate their interaction during host-parasite interaction.,

87 The current decade has seen immense activity in extracellular vesicle research, with  
88 some suggesting that EV release is the main process for modifying the phenotype of  
89 neighboring cells. (Szempluch et al, 2016; Kim et al, 2016; Buck et al, 2014).

90 Physiological release of EVs represents a normal state of the cell ~~raising a metabolic~~  
91 ~~equilibrium or steady state between catabolic and anabolic processes.~~ When the cells  
92 are submitted to stress with different inducers or in pathological situations (malignances,  
93 chronic diseases, infectious diseases, etc), they response with an intense and dynamic  
94 release of EVs (Cocucci & Meldolesi, 2015).

95 Although there is a plethora of literature describing the cargo or content of EVs, there  
96 are very few reports giving an exact description of the mechanism of their release or  
97 providing a clear understanding of the role of EVs in cell-cell communication.

98 The concept that EVs represent intercellular communicative vectors is based on the idea  
99 that the cells release a compartmentalized cargo with proteins, lipids, nucleic acids, and  
100 biomolecules for uptake and integration into other cells. The intense flux between cells  
101 of EVs has been described intensively in recent years in many biological systems and  
102 we have summarized the findings in host-parasite interactions in table 1. With the  
103 increasing research in this field, more information has been obtained in characterization  
104 of EVs by the proteomic analysis of EVs and description of microRNAs contained in

105 exosomes or microvesicles (MVs) from a variety of cell types (Zhang et al, 2015; Eirin et  
106 al, 2014; Alegre et al, 2014). An integrated platform with the data obtained from  
107 proteomics results is available in Vesiclepedia (Kalra et al, 2012).

108 During host-pathogen interaction, protozoan cells employ a vast set of evasion  
109 mechanisms to resist the attack of the immune system to penetrate into the organism  
110 and establish the infection. In recent years EVs have been raised as a new element of  
111 pathogens' evasion mechanisms and modulate parasite-host cell interaction (reviewed  
112 by Evans-Osses et al, 2015; Coakley et al, 2017). In this review, we summarize the role  
113 of EVs in host-parasite interaction in parasite diseases caused by protozoan parasites.  
114 However, our focus is to discuss the application of the knowledge learned in EVs in  
115 different models to develop alternatives to diagnostic, vaccine and translational  
116 medicine.

#### 117 EXTRACELLULAR VESICLES HAVE DIFFERENT MODES OF BIOGENESIS

118 Exosomes and Microvesicles (~~also called microparticles, ectosomes~~) are released from  
119 cells by energy dependent processes display differences in biogenesis, size, function  
120 and cargo. Exosomes (30–100 nm in diameter) are vesicles formed upon inward  
121 budding of endosomes resulting in intraluminal vesicles, within multivesicular bodies;  
122 exosomes are then released by exocytosis within the secretory pathway (reviewed by  
123 Evans-Osses et al, 2015). Exosomes contain proteins derived from their cell of origin  
124 enriched for MHC class I and II, as well as heat-shock proteins and other proteins. MVs  
125 however are not derived from endocytosis, forming instead from a budding of the plasma  
126 membrane, occurring in response to activation of cellular processes (Silva et al, 2017),  
127 in a  $\text{Ca}^{2+}$ -dependent manner, and their size varies (100 nm–1  $\mu\text{m}$ ). Briefly, external  
128 stimuli, such as interaction with pathogen membranes, or some kind of cell damage  
129 result in  $\text{Ca}^{2+}$  influx to the cytoplasm or its release from internal sources. The rise in  
130 intracellular  $\text{Ca}^{2+}$  activates the calpain-mediated cleavage of the actin cytoskeleton.  
131 Flippase and floppase are then inhibited and scramblase is activated transporting the

132 negatively charged phospholipids from the inner to the outer leaflet of the plasma  
133 membrane ( Fujii et al, 2015), resulting in MV formation with phosphatidylserine  
134 exposure. MV content is a reflection of the cellular state (Stratton et al, 2015) and of the  
135 topographic region of the plasma membrane where it was formed (Figure 1). Isolation of  
136 EVs involved in host-pathogen interaction can be performed from several sources, such as cell-  
137 protozoan *in vivo* or *in vitro* interaction/infection and also from parasite axenic culture. In  
138 addition to the *in vitro* approaches, vesicles can be obtained from patients' and from laboratory  
139 animals' biofluids. Researchers use several techniques for the isolation of EVs, the most common  
140 and best accepted being differential centrifugation and size exclusion chromatography (Ramirez  
141 et al., 2017). Detection of EVs is based on their biophysical properties and marker identification.  
142 The most common detection procedures are Western blotting, nanoscale tracking analysis and  
143 electron microscopy (Gardiner et al, 2016). In addition, protein quantification assays can be  
144 used to estimate the protein concentration of EVs.

145 During the long evolution of protozoan species, many pathogens have evolved to invade  
146 a wide range of hosts. Some of these needed to infect host cells to complete the life  
147 cycle and produce an infection. Pathogens need to interact with the immune system  
148 using an array of mechanisms to avoid host immune recognition and effector systems.  
149 The production of EVs is one of these strategies. Protozoan EVs could be involved in  
150 invasiveness, innate recognition, such as the complement system, immunomodulation  
151 and other processes, and this vesicle flux is essential to understand its pathogenicity.  
152 While there is much to be learned about host-parasite interactions, exosomes and MVs  
153 are involved in the persistence of parasite populations within the host.

#### 154 INTRACELLULAR PARASITES

155 *Leishmania sp.*

156 Leishmania parasites, the causative agents of leishmaniasis, are spread by the bite of  
157 phlebotomine sand flies and the disease manifests itself differently in people. The first

suggestion of the release of exosome-like vesicles by *Leishmania* was a description of a large number of known eukaryotic exosomal proteins in *Leishmania* conditioned medium, suggesting a vesicle-based secretion system (Silverman et al. 2008). Later, other authors confirmed these findings (Silverman et al. 2010) showing that the release of leishmanial exosomes is upregulated by infection-like stressors (37°C; ± pH 5.5), also altering the quantity of vesicles and their protein composition. In the same work, the uptake of GFP+ exosomes by infected and non-infected macrophages was observed, and *Leishmania* exosomal proteins HSP70 and HSP90 detected in the cytosol of infected macrophages with specific antibodies. The selective induction of IL-8 secretion in a dose-dependent manner in macrophages treated with exosomes points to an exosomal delivery of molecular messages to infected as well as neighboring uninfected macrophages. It has also been shown that , the protein content of purified exosomes released by macrophages infected with *Leishmania mexicana* promastigotes displays a unique composition and abundance of functional groups of proteins, such as plasma membrane-associated proteins, chaperones and metabolic enzymes compared to the exosomal content of macrophages exposed to LPS and exosomal-free medium (Hassani & Olivier 2013). Macrophages exposed to *Leishmania* release exosomes containing parasite surface protease GP63 that can modulate macrophage protein tyrosine phosphatases and transcription factors in a GP63-dependent manner, playing a notable role in dampening the innate inflammatory response (Hassani et al. 2014). With a different focus, total *Leishmania* RNA was compared with exosomal RNA (Lambertz et al. 2015) and it was shown that exosomes are selective and specifically enriched in small RNAs derived almost exclusively from non-coding RNAs, which could have regulatory functions in cells, influencing host-parasite interactions. The expression and function of an *L. major* phosphatase, LmPRL-1, that participates in the intracellular survival of the parasites inside macrophages was characterized recently (Leitherer et al. 2017) and it has been shown that this protein is secreted mostly inside exosomes .



187 Recognized by WHO as one of the world's 13 most neglected tropical diseases, Chagas  
188 Disease is caused by the protozoan *Trypanosoma cruzi* and represents a relevant social  
189 and economic problem mainly in Latin American countries. *T. cruzi* is able to invade most  
190 eukaryotic cells and has a complex life cycle involving mammalian hosts and insect  
191 vectors (WHO, 2002). The release of EVs has been described in epimastigote,  
192 metacyclic and tissue-derived trypomastigote stages of *T. cruzi*. These EVs may be  
193 released spontaneously or upon activation and they are able to interact with host cells  
194 (Gonçalves, M. F. 1991; Neves, 2014; Silveira, 1979; Cestari, 2012; Bayer-Santos,  
195 2013). Ramirez et al (2017) showed that vesicles obtained by the interaction of parasites  
196 with THP-1 monocytes cells contain components of mutual origin . Analysis of purified  
197 vesicles isolated from the supernatant of infected VERO (African Green Monkey kidney  
198 fibroblast-like) cells indicated that only ~10% of the total proteins detected were of *T.*  
199 *cruzi* origin. Also, it was described that cells infected with metacyclic trypomastigotes  
200 shed vesicles containing GP82, transialidases, gp63, and other parasite proteins  
201 (Bautista-lópez et al. 2017; Bayer-santos et al. 2013). It has been suggested a relevant  
202 role of EVs in interfering with host cell dynamics even before parasite-cell contact is  
203 established. Besides the fact that vesicles isolated from trypomastigote cultures induce  
204 different levels of proinflammatory cytokines and nitric oxide by macrophages in a  
205 heterogeneous manner (Nogueira et al, 2015), the effect of host modulation was shown  
206 in several studies. Microvesicles can also act as an immune evasion mechanism and  
207 result in increased parasite survival. For example, vesicles were shown to form a  
208 complex with C3 convertase, the central enzyme of the complement system, leading to  
209 the decay of the enzyme on the parasite surface. To escape the immune system, *T. cruzi*  
210 could use microvesicles containing TGF- $\beta$ , promoting an increase in the number of  
211 intracellular parasites per cell (Cestari et al, 2012; Ramirez et al, 2017). Interestingly, the  
212 phenomenon of increased infectivity and inhibition of the complement system appears

Comment [J11]: This sentence needs complete rewording. It does not make sense.

Comment [J12]: Please reword. As above.

to be class specific, since vesicles derived from parasites of one class did not alter complement resistance and the invasion process of parasites from the other class (Wyllie & Ramirez, 2017). In vivo, it has been shown that mice previously inoculated with EVs derived from pathogen- host cell interaction from some metacyclic forms, upon receiving a challenge with metacyclic trypomastigotes forms from the same strain of *T. cruzi*, the parasitemia increases in mice (Cestari et al, 2012; Ramirez et al, 2017). However when mice were given an injection of a vesicle fraction prior to *T. cruzi* infection with tissue culture-derived trypomastigotes, this led to increased death and development of a more severe pathology compared to controls (Trocoli Torrecilhas et al, 2009). This consolidates the concept that vesicles induced by parasites contribute to a pro-parasitic environment.

Comment [B3]: Professor, favor modificar.

*Plasmodium sp.*

Malaria is a disease transmitted by *Anopheles* mosquitoes caused by protozoan parasites of the genus *Plasmodium* that infect erythrocyte and hepatocyte cells and was responsible for 212 million new infections and 429 000 deaths worldwide only in 2015. Although malaria can be a deadly disease, illness and death from malaria can usually be prevented (CDC, 2015). *Plasmodium falciparum* parasites directly communicate with other parasites and host cells using exosome-like vesicles that carry parasite proteins and antigens (Martin-Jaular et al, 2011). These are able to deliver genes, as verified by their capacity to transfer drug resistance to parasites (Regev-Rudzk et al, 2013). Extracellular vesicles promote differentiation of gametocytes, the sexual parasite stage for disease transmission to mosquito vector (Regev-Rudzk et al, 2013; Mantel et al, 2013). Extracellular vesicles in *Plasmodium* infection can activate host immune cells and induce macrophage to produce proinflammatory cytokines IL-6, IL-12, and IL-1  $\beta$  and the anti-

240 inflammatory cytokine IL-10 in a dose-dependent manner (Mantel et al, 2013). Red blood  
241 cells parasitized with *Plasmodium* produce 10 times greater numbers of EVs than  
242 unparasitized cells (Nantakomol et al, 2011) and infected patients have higher  
243 frequencies of plasma circulating vesicles compared to healthy controls (Campos et al,  
244 2010). The pathogenic role of EVs was studied *in vivo* in mice during *Plasmodium*  
245 infection, where the peak of plasma EVs coincided with the appearance of neurological  
246 manifestations of cerebral malaria (CM). In this model, fluorescently labelled EVs from  
247 mice with CM were transferred into infected mice and were shown to be attached to the  
248 endothelium of brain vessels. EVs transferred from activated endothelial cells into  
249 healthy recipient mice could induce CM-like histopathological anomalies in brain (El-  
250 Assaad et al, 2014). The relation between EV levels and pathology was demonstrated in  
251 patients by Nantakomol et al (2011), whose group showed that plasma red blood cell-  
252 derived 'microparticle' concentrations were increased in patients with falciparum malaria  
253 in proportion to disease severity. Another study found increased numbers of circulating  
254 endothelial-derived vesicles in children with coma and severe malaria. In this work it was  
255 also noticeable that during convalescence of the infection the number of endothelial-  
256 derived vesicles was significantly less than in the acute stage among patients with  
257 cerebral malaria or coma and severe anemia (Combes et al, 2004).

258

#### 259 *Toxoplasma gondii*

260 Toxoplasmosis is caused by *Toxoplasma gondii*, an intracellular protozoan that  
261 infects most nucleated cells found worldwide. *T. gondii* infection may be asymptomatic in  
262 immunocompetent individuals, but may cause severe, life-threatening illness in  
263 immunocompromised individuals and fetal complications if the mother experiences  
264 primary infection during pregnancy (Beauvillain et al. 2009). Knowledge about exosomes  
265 secreted by *T. gondii*, or by cells infected by the protozoan is still very limited (Dlugonska  
266 & Gatkowska, 2016; Wowk et al, 2017). Pope & Lässer (2013) hypothesize that

267 exosome-like particles derived from *T. gondii*-infected fibroblasts could participate in the  
268 pathogenesis of the parasite, and could be responsible for increasing mRNA levels  
269 associated with neurological activity (Rab-13, thymosin) and their transfer to uninfected  
270 cells. The first proteomic profile from EVs of *T. gondii*, obtained from infected human  
271 foreskin fibroblast, reveals biomolecules already described in other models, such as  
272 CD63, HSP 70, and calcium-binding proteins (Wowk et al, 2017). Kim et al. (2016)  
273 observed changes in the proliferation of myoblasts treated with exosomes derived from  
274 infected cells (increase in the S phase).

275

## 276 EXTRACELLULAR PARASITES

### 277 *Giardia intestinalis*

278 *G. intestinalis* is one of the most prevalent gastrointestinal pathogens on the planet that  
279 produce a diarrhoea with low inflammation and in some cases a chronic infection. The  
280 replicative form trophozoites need to adhere to the small intestine mucosa for survival.  
281 Secretory products involved in the communication between leukocytes by parasites was  
282 reported by Lee et al (2012): Trophozoites of *G. intestinalis* stimulated the production  
283 of IL-8 which was responsible for the recruitment of neutrophils. As reviewed by Evans-  
284 Osses et al (2015), it is suggested that the EVs represent an important form of  
285 communication and immunomodulation. Evans-Osses et al. (2017) identified the release  
286 of MVs from *G. intestinalis*, for the first time. The release of MVs may vary at different  
287 pH conditions (7.0 being the best condition) and time, and their release increases the  
288 adhesion capacity of the protozoa in Caco-2 (Caucasian colon adenocarcinoma cells)  
289 and their uptake and maturation by human dendritic cells. The pathogenesis of *Giardia*  
290 is not yet fully understood. Kho et al (2013) demonstrated that HCT-8 (ileocecal  
291 colorectal adenocarcinoma cells) infection could induce apoptosis, with signs of  
292 chromatin condensation and activation of caspase-3. It was demonstrated that extracts

293 of parasites in contact with Caco-2 induced apoptosis, showing that the presence of the  
294 parasite is not necessary to start the process and suggesting that it could be dependent  
295 on EVs.

296

#### 297 *Trypanosoma brucei*

298 Nten et al (2010) identified for the first time that secretomes of *T. brucei* (*T. brucei brucei*,  
299 *T. brucei gambiense*) are associated with the pathway of exosomal biogenesis, being  
300 composed of proteins associated with pathological processes and the evasion of the  
301 immune system (metallopeptidases, Gp63 protease). Eliaz et al. (2017) demonstrated  
302 that exosome secretion was inhibited in *T. brucei* when transcription is inhibited (*Vps 36*),  
303 but production of exosomes continued in nanotubes. In addition, EVs could interfere in  
304 the social motility of parasites, repelling individuals from unfavorable conditions. Using  
305 time-lapse microscopy, it was demonstrated that exosomes were secreted for the  
306 transmission of stress signals. Szempruch et al (2016) concluded that exosomes contain  
307 mostly flagellar and membrane proteins, such as surface glycoproteins and HSP-70.  
308 These particles merge with erythrocytes, causing a decrease in circulation, and possibly  
309 resulting in host anemia. Furthermore, *T. brucei rhodesiense* can transfer vesicles  
310 containing serum resistance-associated proteins to non-human trypanosomes, allowing  
311 immune system evasion. A number of proteins contained in exosomes of *T. brucei*  
312 *gambiense* (HSP-70, RAB protein,  $\alpha$  and  $\beta$  tubulins, heavy chains of clathrin, histamine)  
313 have been identified (Geiger et al. 2010), with physiological functions not only for survival  
314 in the host, but also for immunomodulation and intercellular communication. Enzymes  
315 involved in nucleotide metabolism were also identified, which could influence the  
316 inflammatory process.

317

#### 318 *Trichomonas vaginalis*

319 *Trichomonas vaginalis* colonizes the human urogenital tract producing sexually  
320 transmitted disease known as trichomoniasis. Twu et al. (2013) identified for the first time  
321 the participation of *T. vaginalis* exosomes in immunomodulation and host-parasite  
322 communication, allowing an improvement of adhesion when exosomes from highly  
323 adhesive parasites were incubated with a less adhesive parasite. Olmos-Ortiz et al  
324 (2017) observed the immunomodulatory effects of *T. vaginalis* exosomes on murine  
325 macrophages. These vesicles induced an increase of IL-10, IL-6, TNF- $\alpha$  expression, and  
326 nitric oxide production (cytotoxic and immunomodulatory activity). In addition, infected  
327 mice treated with exosomes increased IL-10 production and decreased IL-17 levels,  
328 resulting in the diminution of the inflammatory process without a reduction in parasitic  
329 load.

330

#### 331 WHAT IS THE NEXT STEP: TRANSLATIONAL APPLICATIONS.

332 The apparent role of EVs in a large number of biological processes, along with many of  
333 their intriguing features, forms the basis of extending EV analysis beyond basic research  
334 and into the clinical and therapeutic context (Revenfeld et al, 2014). EV isolation methods  
335 should be more specific, to ensure safe therapeutic possibilities (Momem-Heravi et al,  
336 2014; Alvarez-Erviti et al, 2011; Zhou et al, 2013). In this regard, the development of  
337 portable point-of-care diagnostic tools for detecting circulating exosomes as biomarkers,  
338 should be important in the future. Although much effort has been employed to  
339 understand the biology of EVs, we still need more experimental advancements to  
340 develop applied methods for the community. The need for such diagnostic tools in  
341 developing countries are very important as many are burdened by parasitic diseases,  
342 and lack professional and material resources.

343 While naturally secreted exosomes may mediate beneficial effects in certain disease  
344 conditions, targeted exosomes loaded with therapeutic molecules may optimize efficacy  
345 while also reducing off-target delivery (Barile & Vassali, 2017; Moore et al., 2017) The

lipid composition of their membranes may also increase antigenic stimulation (Zitvogel et al, 1998; Escudier et al, 2005). It is now imperative that the findings from basic research are translated into biotechnological applications with greater urgency (Fontana et al, 2012).

## *Vaccines*

Among numerous biomedical applications, the use of EVs in immunization may be explored in the future. The way EVs interact in antigen presentation allows the possibility of its use in developing a T cell-dependent response. In the past decade, it is laudable that philanthropic initiatives have been involved in the production of vaccines, since diseases caused by unicellular eukaryotes are a major burden to tropical developing countries.

The genome of many protozoa has already been studied, and others have been initiated for the study of transcriptomes (Birkeland et al, 2010; Rastrojo et al, 2013; Morse et al, 2016). However, for ethical reasons, it is not possible to use live cell lines in an immunization protocol in humans (Beauvillain et al, 2009), especially considering the contraindication of live attenuated vaccines for immunocompromised patients.

Exosomes could therefore provide a new method for communication and for the exchange of antigenic information between cells in the immune system (Aline et al, 2004). Since they are of the same size as viruses, a similar uptake by antigen-presenting cells may be observed. Interestingly, the use of exosomes as a versatile tool for signal delivery compared with soluble molecules is gaining support due to their double-layered membrane (Trelis et al, 2016). In eukaryotic pathogens, both immuno-stimulatory and immuno-inhibitory functions have been reported for exosomes (Atayde et al, 2016). The ability of exosomes, especially those derived from dendritic cells (DCs), to induce protective immune responses offers an alternative to DC-based vaccines (Beauvillain et

al, 2007). Dendritic cells, presenters of antigens that participate in the onset of the adaptive response, are able to secrete EVs carrying MHC class II antigens, allowing the development of a specific T cell response; these EVs would be antigen-presenting vesicles (Zitvogel et al, 1998; Escudier et al, 2005).. Intracellular parasites, bacteria, and viruses that enter cells via an endocytotic pathway are prime candidates for DC-based exosome Immunogens. Vaccines consisting of exosomes will both preserve the positive aspects of live parasite vaccines and avoid their inherent risks (del Cacho et al, 2012). Aline et al (2004) demonstrated for the first time the participation of exosomes in protection against pathogens. They developed a vaccine composed of exosomes of DCs stimulated by *T. gondii*, capable of eliciting a TH1-mediated response. This protective capacity may be associated with DCs or exosome trafficking. Dendritic cells stimulated *in vitro* with *T. gondii* antigens secreted exosomes capable of inducing a significant humoral responses in syngeneic and allogeneic mice, with a greater participation of IgA and reduction of cysts in the brain. Due to the low safety of administering an attenuated *T. gondii* vaccine to humans (Beauvillain et al, 2007), the use of antigen-presenting vesicles is a possible way of stimulating T cells.

Exogenous derivatives of DCs, stimulated with *Leishmania major* antigens, created a protective response with TH1 activation against cutaneous leishmaniasis (Schnitzer et al, 2010).

Martin-Jaular et al (2011) verified increased IgG in mice by treating them with exosomes of reticulocytes infected with *Plasmodium yoelli*. Antibodies were able to recognize erythrocytes infected with the protozoan. A summary of EVs involved in immunity is shown in Table 2. A summary of EVs involved in immunity is shown in Table 2 and some of possible strategies are illustrated schematically in Figure 2 and 3.

However, although the use of exosomes allows for a cell-based vaccine, there are both conceptual and practical issues that need to be addressed before this potential application can become a reality. These include obtaining exosomes with the correct mix



of antigens that provide protection, with the risks of introducing 'non-self' human molecules into a vaccinated individual (Schorey et al, 2015). Through EVs, protozoa are able to initiate proinflammatory responses from target cells, such as increased production of interleukins. Therefore, it is suggested that the vesicles may have the added benefit of acting as adjuvants. Exosomes have also been shown to induce anti-tumor immunity in the absence of adjuvants or heat treatment (Greening et al, 2015; Morishita et al, 2016) thus suggesting the use of exosomes as adjuvant carriers because of the ability of these structures to act as molecule carriers. Due to their physical properties, EVs could enhance the immunogenicity of antigens. A satisfactory response must activate specific arms of the immune system, such as the cell-mediated response, and possibly constitute an effective immunomodulatory effect for diseases that lack an effective immunogen.

#### *Diagnostic*

The level of EVs in human serum could be a marker of disease status (Pisitkun et al, 2004; Wekesa et al, 2014; Kim et al, 2017). As EVs reflect the proteomic content of the cells from which they are derived, it is possible to use them for disease detection, as first investigated in tumors (Santiago-Dieppa et al, 2014). EV cargo can also be involved in disease prognosis. The nature of the vesicles allows a means of transport, free of blood degradation. This has proven to be particularly significant for the use of miRNA as valuable biomarkers because most RNA in blood exists within EVs (Revenfeld et al, 2014). Recently, Melo et al (2015) detected cancer-cell derived exosomes containing a high concentration of cell surface proteoglycan, glypican-1. It was showed that glypican-1 is a pan-specific marker of cancer exosomes, specifically detected in the serum of individuals with pancreatic cancer. Many parasitic diseases have parasite-host interactions with poorly studied pathogenesis. As EVs participate in these processes, clinical investigation should consider the detection of EVs derived from parasites or hosts

428 in biological fluids. When the *T. cruzi* secretome was analyzed after 16 hours of  
429 ultracentrifugation, proteins involved in the pathogenicity of the protozoa, such as GP63  
430 and Aminopeptidase P, were found to be increased within the EVs compared to the  
431 supernatant fraction (Bayer-Santos et al, 2014). According to Geiger et al. (2010)  
432 metallopeptidase thimet oligopeptidase A (M3 family) is the first of the group to be  
433 identified in Protozoa, resulting in a potential marker for diagnosis in *T. brucei*. The  
434 presence of proteins on the EV lipid bilayer such as the tetraspanins, could present  
435 targets for detection by monoclonal antibodies .

436 It is also possible that nucleic acid ,present in EVs has some diagnostic potential (Melo  
437 et al, 2015;, Zhang et al, 2015; Eirin et al, 2014). Such a molecular diagnosis of parasitic  
438 diseases offers high sensitivity and specificity, compared to microscopic examination,  
439 reducing shortcomings and it can be standardized (Stensvold et al, 2011). As microscopy  
440 remains labour intensive and highly observer-dependent, molecular detection  
441 techniques, such as real-time PCR, are excellent alternatives, in particular in settings  
442 where the number and range of parasitic infections is low and personnel costs are  
443 substantial (Lieshout & Roestenberg, 2015). Serological assays also have limitations.  
444 For example, conventional serological assays for *T. cruzi* may lead to unspecific  
445 reactions (false positives) due to cross reactivity with antibodies elicited by other  
446 pathogens, e.g., *Leishmania* spp. and *Trypanosoma rangeli*. Because of a lack of a  
447 single test with high sensitivity/specificity, the WHO recommends positive results from  
448 two different serological tests for a confirmation of *T. cruzi* infection (Zingales, 2017). In  
449 that context, diagnostic techniques based on molecular targets are becoming more  
450 popular.

451 Accordingly, as reviewed by Inamdar et al (2017), expression profiling can be useful as  
452 a diagnostic tool in diseases that lack definitive biomolecular biomarkers. The detection  
453 of nucleic acids in EVs obtained from clinical samples, like feces, could become a regular  
454 procedure . It was shown by Liu et al, (2016) that is possible to obtain a suitable Fecal

Suspension Particle size Distribution by NanoSight and EM Feces from mice with a combination of dilution of feces in PBS, spun down at 10,000 ×g for 5 min by centrifugation and then filtered to remove the debris. Moreover, EVs were isolated from feces homogenized in PBS, using exoEasy Maxi Spin Columns (Qiagen), followed by RNA isolation using miRCURY RNA isolation kit (Exiqon) with on-column DNase treatment (Qiagen).

Other types of analytes that could be studied in the exosomal space, such as lipids, might represent good biomarkers to investigate in the near future. All these provide an important base to continue research in parasite-derived exosomes as diagnostic targets and demonstrating their utility as clinical biomarkers (Sánchez-Ovejero et al, 2016).

#### *Therapeutics*

Extracellular vesicles participate in cellular traffic thereby influencing pathophysiological conditions and it is this capacity to be delivered that could be extrapolated for therapeutic purposes as well (Moore et al., 2017). Mammalian stem cells EVs have been shown to be involved in cell proliferation and improved renal function of cisplatin-induced acute kidney injury in rats (Zhou et al, 2013). For the same model, it was also demonstrated that exosomes could deliver miRNA for up-regulation of anti-apoptosis genes, and reduce mortality caused by cisplatin (Bruno et al, 2012). Intranasal delivered exosomes were taken up by microglial cells, which are key mediators in neuro-inflammatory diseases; delivery of curcumin-loaded exosomes resulted in a reduction in activated microglial cells in both encephalitis and LPS-induced brain inflammation models (as reviewed by Inamdar et al, 2017).

There are different ways to explore the potential of therapeutic EVs. For example, macrophage-derived MVs may represent a way of converting an autologous intrinsically biocompatible sub-cellular entity into a drug delivery system able to carry both nanoparticles and drugs. In this regard, it would be of interest to demonstrate that cellular

481 MVs might be loaded with different drug molecules while simultaneously enclosing  
482 nanoparticles that enable spatially controlled drug delivery.

483 Silva et al (2015) encapsulated different drugs with magnetic nanoparticles within MVs.  
484 The magnetic properties of the nanoparticles could influence the uptake of the loaded  
485 MVs, and such a combination could represent a model for the delivery of different types  
486 of drugs, as for example in cancer-therapy. The possibility of producing MVs from  
487 different cell populations could improve such association, resulting in a specific delivery  
488 method. Strategies for loading molecular cargo in exosomes and related efficacies differ  
489 based on the chemistry of the loaded molecule (as reviewed by Inamdar et al, 2017). In  
490 this regard, it is possible to explore the loading capacity of EVs for different antiprotozoal  
491 drugs.

492 Besides drug delivery, nucleic acids have also been involved in therapeutic purposes.  
493 The specificity imparted by targeted exosomes, the ability to load exogenous genetic  
494 cargoes, the ability to systemically administer the gene therapy and immune evasion by  
495 exosomes are valuable properties for future oligonucleotide therapy applications  
496 (Alvarez-Erviti et al, 2011). In addition, exosome-transferred miRNAs are emerging as  
497 novel regulators of cellular function (Alexander et al, 2015). Momem-Heravi et al (2014)  
498 loaded exosomes with microRNA (miR-155, with electroporation) or inhibitor to be  
499 uptaken by hepatocytes or macrophages, respectively. Exosome-mediated inhibition  
500 was superior to conventional transfection models. Alvarez-Erviti et al (2011) through *in*  
501 vivo administration of exosome-loaded siRNA were able to specifically knockdown BACE  
502 1 (protease involved in Alzheimer's Disease).

503 Delivery of drugs or nucleic acids through EVs to treat parasitic diseases have not been  
504 explored yet. However, mammalian models shows promise. We speculate that if EV-  
505 loaded microRNAs inhibit genes involved in the pathogenesis of protozoa, it will  
506 significantly affect parasite-host interactions. MicroRNA could specifically regulate  
507 protozoan virulence through inhibition of invasiveness-related genes, or improve the host

Comment [IR5]: Especulate

immune system by up-regulating immune-related genes. Another promising strategy relies on gene-editing tools, like CRISPR/Cas 9. CRISPR/Cas 9 is a technology based on a known mechanism from bacteria and archaea that enable the organisms to respond to and eliminate invading genetic material. The CRISPR/Cas9 system consists of the Cas9 nuclease and a single guide RNA, which are used to guide effector endonucleases that target interested DNA sequence based on sequence complementarity (Chira et al, 2017). While *in vivo* delivery of this system has a low efficiency, the use of exosomes loaded with CRISPR/Cas9 showed a promising result in cancer. Kim et al (2017) demonstrated a reduced apoptosis in ovarian cancer by suppressing poly (ADP-ribose) polymerase-1, with CRISPR/Cas9 loaded exosomes. While there is a long way in medical approaches concerning the application of this tool, a CRISPR/Cas9 system editing pathogen genes will allow a better understanding about host-pathogen interaction. This knowledge could provide the possibility of designing novel targets for therapeutic interventions .

#### CURRENTL REMARKS

The strategies debated in this work are speculations of what is to come from the rapidly expanding field of EVs. It is clear that “trial-and-error” research is necessary to expand applications in the routine medical laboratory. Since parasitic diseases are common in developing countries, translation into clinics must involve low-cost strategies. If participation of EVs in cell communication models are becoming highly proven, it is only a matter of time until biotechnology is able to deliver accessible procedures.

Ar EV-detection methods going to emerge as markers for next-generation diagnostics of protozoa?

Are EVs a satisfactory alternative for the immunization of poorly responsive populations?

534 Can EVs be considered a delivery system for drugs to reduce off-target effects on  
535 parasitological diseases?

536 Is Clinical parasitology going to translate extracellular vesicle-based research in the  
537 future?

538 Can gene-editing systems interfere in host-parasite communication, acting as a  
539 therapeutic alternative?

540

541

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543

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Comment [J16]: Some Refs have d.o.i. Others do not. I guess it is more likely that this is not required.

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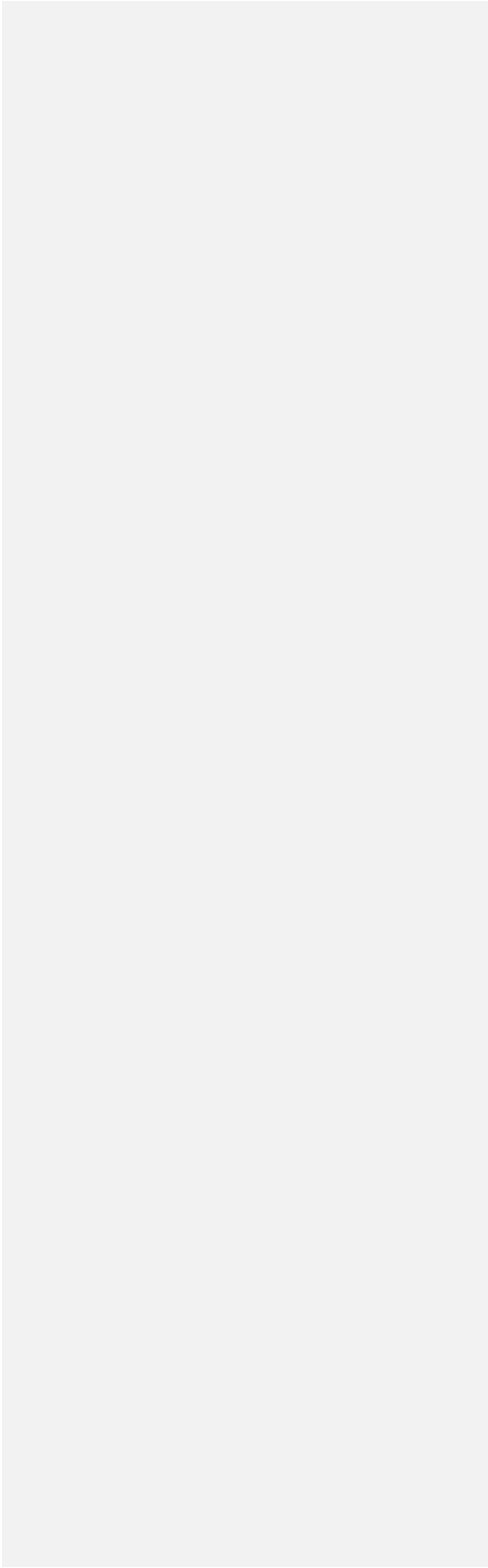
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963 Figure Legends

964 Figure 1. Schematic representation of the dynamic flux of extracellular vesicles

965 between host cells and parasites.

966 Figure 2. Different strategies to use EVs as vaccines and trigger antibodies production.

967 Figure 3. Loading surface molecules into nanovesicles. Vesicles coupled with antigens or

968 carrying specific antibodies acting as vaccines or neutralizing pathologic effects.

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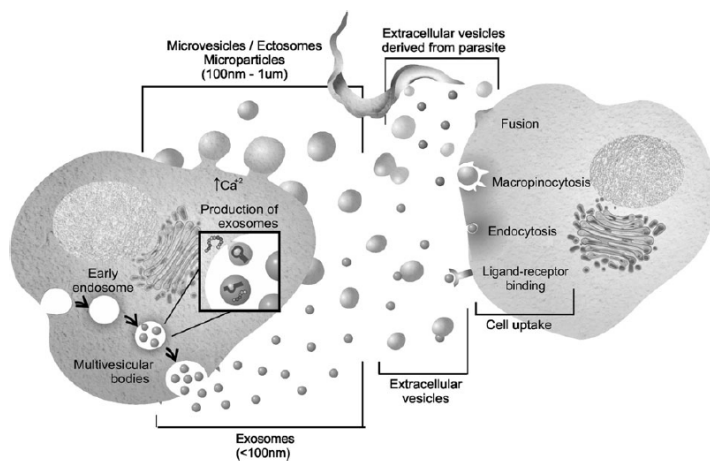


Fig. 1. Schematic representation of the dynamic flux of extracellular vesicles between host cells and parasites.

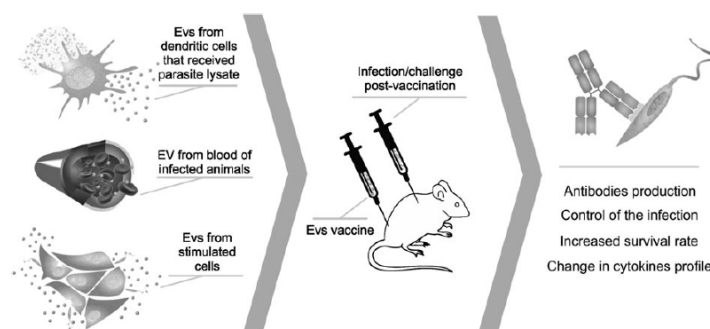


Fig. 2. Different strategies to use EVs as vaccines and trigger antibodies production.

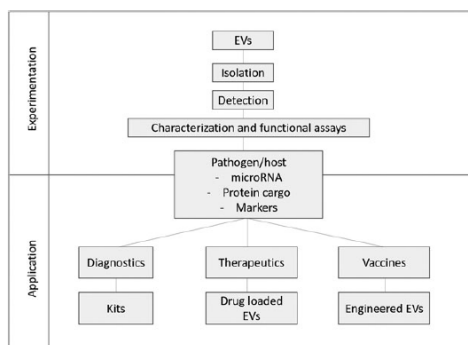


Fig. 3. Experimental steps for the Study of EVs and the possibilities of translation into clinics.